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Prolonging bovine sperm–oocyte incubation in modified medium 199 improves embryo development rate and the viability of vitrified blastocysts

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Abstract

This study was conducted to compare the efficacy of four in vitro fertilization (IVF) media: Bracket and Oliphant's medium (BO), modified medium 199 (IVF-M199), modified Tyrode's medium (MTM), and modified KSOM (m-KSOM) on fertilization efficiency and blastocyst formation rate. In addition, we wanted to investigate the benefit of prolonging the IVF period (from 6 to 18 h) using the two most effective IVF media determined in our initial experiment; subsequently, blastocyst viability was assessed following vitrification. A higher incidence of polyspermic fertilization was observed in the MTM (6%) and in BO, in both the 6 and 18 h (7% and 11%, respectively) groups, than in the m-KSOM (1%) or in the IVF-M199 6 or 18 h (1 and 3%, respectively) groups. Cleavage rates were similar in BO, IVF-M199, and MTM 48 h post-fertilization; however, the lowest cleavage rate was observed for m-KSOM. A greater proportion of zygotes developed into 8-cell embryos in IVF-M199 than in other IVF media. Subsequently, a greater proportion of blastocyst formation and hatching was achieved in IVF-M199 (40% and 79%, respectively) or BO (35% and 74%, respectively) than in m-KSOM (18% and 58%, respectively) or MTM (22% and 66%, respectively). Prolonging IVF to 18 h did not alter cleavage rates; however, the highest rate of overall blastocyst formation was achieved in the IVF-M199 18 h (49%), rather than in the BO 18 h (20%) group. Vitrified/thawed blastocysts from IVF-M199 groups re-expanded and developed better, as compared to the BO 18 h group, and hatching rate and total cell number in IVF-M199 18 h group was comparable to the control groups (non-vitrified). Vitrification reduced survival compared to controls. In conclusion, IVF-M199 was successfully used for IVF, compared favorably to BO medium, and offered the advantage of an extended IVF period for up to 18 h that requires only one-half a dose of semen, and resulted in better quality blastocysts that endured vitrification with a hatching rate comparable to that of control groups.

Keywords: Fertilization; IVF-M199; BO; Vitrification; Bovine

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1. Introduction

To date, IVF media have been consistently formulated with the distinct goal of promoting favorable conditions for capacitating sperm to fertilize matured oocytes. However, some IVF media may not be suitable for incubating oocytes with sperm for a prolonged IVF period. As a result, IVF methodology usually employs a short (6 h) incubation period of bovine oocytes with frozen-thawed sperm, in varying high ionic strength (HIS) fertilization media, e.g. Bracket and Oliphant's medium (BO) and modified Tyrode's medium (MTM) [1–4]. The standard 6 h sperm–oocyte incubation period is widely used in research studies [1]. However, it is less common in commercial IVF programs, mainly because of the inconvenience and expense of extended work schedules and overtime pay. Moreover, it has been recommended that it is advantageous to remove oocytes as soon as possible from suspensions of dead or dying sperm in BO medium [4]. This limits the cost effectiveness and convenience of prolonging incubations to accommodate a normal work schedule. Effectively lengthening the incubation period for oocytes and sperm, without compromising fertilization efficiency, would overcome these obstacles; however, it appears that extending IVF periods produces high levels of reactive oxygen species (ROS), generated by dying sperm in fertilization drops, which results in zona hardening and affects an embryo's developmental viability [5]. Thus, the media currently utilized, for both the washing of frozen-thawed sperm following cryopreservation, and for IVF, although much improved in recent years, is not entirely satisfactory and does not effectively replicate in vivo conditions.

In vitro fertilization medium must play a critical role, not only for the separation of frozen-thawed motile sperm from their cryoprotectants, but also in providing conditions meeting the appropriate physiological requirements for metabolic activities of both sperm and oocyte during IVF incubation. In vivo, these conditions are provided by the female's reproductive tract, whereas in vitro these need to be mimicked by IVF medium [6,7]. Consequently, formulating sperm wash and fertilization medium in vitro necessitates including components or chemicals that promote sperm motility, capacitation, the union of two gametes, and the initiation of embryo development; these usually include: caffeine [8], heparin [9], caffeine plus heparin [10], glucose [11], calcium ionophore A23187 [12], bicarbonate [13–15], and inositol [16]. These chemicals have been shown to be essential for a successful fertilization in vitro. Simple media with high ionic strength, such as BO [4], has been widely utilized for both washing frozen-thawed semen, and subsequent IVF. This medium, however, contains only a few components, and may cause accelerated oocyte aging that can be a factor in permitting fertilization by multiple sperm (polyspermy), and it reduced embryonic development if used for a prolonged (18 h) IVF interval [2]. In contrast, commonly used oocyte maturation medium, such as medium 199 (M199), and embryo culture medium, such as KSOM, are highly suited for oocytes and embryo development. The use of these media during fertilization, therefore, may be beneficial to both the oocyte and sperm; although, without modification, M199 or KSOM lack specific components important for sperm activation, fertility, and motility.

In this study, we aimed to compare four different routinely utilized IVF media for their effect on fertilization and on embryonic development rate to blastocyst stage. Furthermore, we were interested in testing the effects of the duration of sperm–oocyte incubation (6 h versus 18 h), in these media because increasing the sperm–oocyte incubation period would be highly desirable, and our modified IVF media were not expected to cause oocyte damage.

2. Materials and methods

All chemicals used in this study were purchased from Sigma Chemical Co. (St. Louis, MO, USA), unless otherwise indicated.

2.1. Bovine oocyte collection and maturation in vitro

Bovine oocytes were aspirated from ovaries collected at an abattoir (Yankton, SD, USA), and matured in Tissue Culture Medium199 (IVM-M199) with Earle's salts, L-glutamine, 26.19 mM sodium bicarbonate, and 25 mM HEPES plus 10% fetal bovine serum (FBS, Hyclone, Logan, UT, USA), 1% antibiotic/antimycotic (Gibco, Grand Island, NY, USA) and 10 ng/mL epidermal growth factor (EGF). Briefly, cumulus-oocyte complexes (COCs) were placed in sterile cryovials containing pre-gassed maturation medium (IVM-M199) and shipped to the laboratory overnight in a portable incubator (Minitube of America, Verona, WI, USA) at 38.5 °C. On arrival, oocytes were cultured in 5% CO_2 in air at 39 °C until the total time for maturation reached 24 h.

2.2. In vitro fertilization (Experiment 1)

2.2.1. Preparation of sperm wash and in vitro fertilization media

All sperm wash media were made from modified BO's fertilization media [1]; BO's fertilization and sperm wash medium was described in detail by Brackett and Oliphant [4]. Our media preparations are briefly described here. (1) BO's fertilization medium contained 6 mg/mL of fatty acid-free bovine serum albumin (FAF-BSA) and 10 μg/mL heparin. For sperm wash, the heparin was omitted and 5 mg/mL BSA fraction V and 10 mM caffeine were added; (2) modified M199 (IVF-M199, M2520; with Earle's salts, L-glutamine and buffered with 25 mM Hepes) was supplemented with 37 mM Sodium bicarbonate, 1% antibiotic/antimycotic (Bio-Whittaker, Walkersville, MD, USA), 13.9 mM Glucose, and 1.25 mM sodium pyruvate. The IVF-M199 contained 6 mg/mL FAF-BSA, and 10 μg/mL heparin. Sperm wash medium included: IVF-M199, 5 mg/mL BSA fraction V, and 10 mM caffeine; (3) potassium simplex optimization medium [17] was modified (m-KSOM) for in vitro fertilization by adding 13.9 mM glucose, 1.25 mM sodium pyruvate, 1% antibiotic/antimycotic, 6 mg/mL FAF-BSA and 10 μg/mL heparin. The m-KSOM sperm wash solution contained 5 mg/mL BSA fraction V and 10 mM caffeine; (4) modified Tyrode's medium (MTM fertilization medium was described previously [18–20]). The MTM used for fertilization contained 6 mg/mL of FAF-BSA and 10 μg/mL heparin. The MTM sperm wash used in this study was described in detail by Parrish et al. [21] with the addition 5 mg/mL BSA fraction V. Components before and after supplementation of the four IVF media are shown in Table 1.

2.2.2. In vitro fertilization procedure

Following 24 h of maturation in vitro, bovine COCs were removed from maturation medium (IVM-M199) and washed three times in TL-Hepes (Bio-Whittaker, Walkersville, MD, USA), and aliquoted into groups of 20 to 25. Then, they were washed three times in their assigned fertilization medium (BO, IVF-M199, m-KSOM, or MTM) and transferred into 50 μL drops of their respective fertilization medium in a Petri dish and placed under mineral oil (Becton Dickinson, Franklin Lakes, NJ, USA). The dishes were maintained at 5% CO_2 in air at 39 °C. One straw of semen (ABS, DeForest, WI, USA) was thawed at 38 °C for 1 min and divided equally into four aliquots for the sperm wash media trials (BO, IVF-M199, m-KSOM, or MTM), and semen from only one bull was used throughout. Using the appropriate one of each of the four sperm wash media, the sperm were washed two times by centrifugation at 453 × *g* for 8 min. Subsequently, sperm pellets were re-suspended in the appropriate sperm wash media to a volume of 250 μL. Following the final wash, sperm motility, and concentration was determined. Thirty microliters (30 μL) of the re-suspended sperm was added to each fertilization drop, giving a total concentration of 1×10^7 sperm/mL in each of the four IVF media tested. Oocytes were then incubated with the washed sperm for 6 h in 5% CO_2 in air at 39 °C.

2.2.3. Confirmation of fertilization and culture of presumptive zygotes

After 6 h of in vitro fertilization, sperm attached to cumulus cells surrounding oocytes (in each treatment group) were washed by votexing for 2 min in 200 μL TL-Hepes in 1 mL eppendorf (Fisher, Pittsburg, PA, USA). Thereafter, 10 presumptive zygotes were collected from each of the fertilization medium groups and fixed (replicated 8 times, $n = 320$) to evaluate pronucleus formation and polyspermy. The fixation solution was acetic alcohol (acetic acid: ethanol, 1:3, v/v) and the nuclear stain was 1% aceto-orcein in acetic acid [22]. Oocytes with two pronuclei (PN-2) were considered to have been fertilized normally; those with three or more pronuclei (≥PN-3) were considered to be polyspermic, and the remaining oocytes were considered not penetrated.

All remaining presumptive zygotes (total, $n = 1335$) were cultured in our previously described sequential KSOM-SOF culture system. Briefly, embryos were cultured for 4 days in 25 μL of KSOM plus 0.1% BSA, and then moved into SOF + 5% FBS, and cultured until Day 9, in 5% O_2, 5% CO_2 and 90% N_2 at 39 °C. The day of fertilization is designated as Day 0. Embryo cleavage rate was recorded 48 h post-fertilization, total blastocyst on Day 8, and hatched blastocysts on Day 9.

2.3. Blastocyst formation following short (6 h), or prolonged (18 h) oocyte and sperm co-incubation in BO medium or IVF-M199 (Experiment 2)

Based on the results from Experiment 1, the two best IVF media (BO and IVF-M199) were selected for short (6 h), or prolonged (18 h) sperm–oocyte incubation periods, and the embryo viability was examined after vitrification/thawing. Standard and reduced concentrations of sperm (1×10^7 and 5×10^6 sperm/mL) were used for a standard 6 h or an extended (18 h) IVF incubation period. Briefly, BO medium or IVF-M199 was used in this experiment for sperm wash and in vitro

Table 1
Components of IVF media

Components	BO	M199	IVF-M199	KSOM	m-KSOM	MTM
Caffeine (mM)	10	–	10	–	10	10
Heparin (μg/mL)	10	–	10	–	10	10
Glucose (mM)	13.9	5.6	13.9	0.2	0.35	–
Glutathione (GSH) (mM)	–	0.002	0.002	–	–	–
L-Ascorbic acid (LAA) (mM)	–	0.003	0.003	–	–	–
$NaHCO_3$ (mM)	37	–	37	25	25	25
$CaCl_2{\cdot}2HO_2$ (mM)	2.25	1.36	1.36	1.71	1.71	2.10
NaCl (mM)	112	103	103	95	95	100
BSA fraction V (sperm wash medium) (mg/mL)	5	–	5	–	5	5
BSA-FAF (A-6003, IVF medium) (mg/mL)	6	–	6	–	6	6
KCl (mM)	4.02	5.37	5.37	2.50	2.50	3.2
KH_2PO_4 (mM)	–	–	–	0.35	0.35	–
L-Glutamine (mM)	–	0.68	0.68	1.00	1.00	–
EDTA (mM)	–	–	–	0.01	0.01	–
Hepes (mM)	–	25	25	2.50	2.50	10
$NaH_2PO_4{\cdot}H_2O$ (mM)	0.83	0.88	0.88	–	–	0.34
$MgSO_4{\cdot}7H_2O$ (mM)	0.52	0.4	0.4	0.20	0.20	1.5
Na-lactate (C_3H5O_3) (mM)	–	–	–	10	10	10
Na-pyruvate (mM)	1.25	–	1.25	0.20	0.20	
Sodium acetate anhydrous (CH_3COONa) (mM)	–	0.61	0.61	–	–	–
Taurine (mM)	–	–	–	0.40	0.40	–
Myo-inositol ($C_6H_{12}O_6$) (mM)	–	0.0003	0.0003	–	–	–
Phenol red (μg/mL)	10	–	–	10	10	10
Antibiotic (100×) (μL/mL)	10	10	10	10	10	10
EAA (50×) (μL/mL)	–	–	–	10	10	–
NEA (100×) (μL/mL)	–	–	–	10	10	–

fertilization. A total of 1037 matured oocytes were randomly allocated among 4 treatment groups: BO 6 h ($n = 260$), BO 18 h ($n = 258$), IVF-M199 6 h ($n = 253$), and IVF-M199 18 h ($n = 266$). Sperm preparation procedures were described in Experiment 1. After sperm–oocytes incubated for 6 or 18 h in BO or IVF-M199, 10 presumptive zygotes per replicate (8 replicates) were fixed and stained ($n = 320$) for polyspermic analysis, as described in Experiment 1. The remaining presumptive zygotes were cultured in KSOM-SOF culture system. The blastocysts which resulted from oocytes fertilized in BO or IVF-M199 for 6 or 18 h were tested for their viability after being subjecting to solid-surface vitrification.

2.3.1. Vitrification of blastocysts produced from BO or IVF-M199

Expanded-blastocysts, generated from matured oocytes fertilized following a 6 or 18 h incubation period in BO medium or IVF-M199 were vitrified by a solid-surface vitrification (SSV) method. Briefly, M199 with Earle's salt buffered with 25 mM Hepes supplemented with 20% FBS (Hyclone, Logan, UT, USA) was used as a basal medium, and an equilibration solution was prepared by adding 10% (v/v) ethylene glycol (EG) in M199 + 20% FBS. The resulting modified solid surface vitrification (mSSV) method contained 35% EG + 0.5 M sucrose + 5% (w/v) PVP in M199 + 20% FBS.

One half of Day 7 expanded-blastocysts were equilibrated in two steps; these were derived from four groups, including matured oocytes fertilized in BO for 6 h, those fertilized in BO for 18 h, those fertilized in IVF-M199 for 6 h, and those fertilized in IVF-M199 for 18 h. Briefly, blastocysts were washed and equilibrated in holding medium (M199 + 20% FBS) for 10 min in an incubator with 5% CO_2 (39 °C), and subsequently equilibrated in 10% EG for 5 min on a warming plate (39 °C). Following equilibration, blastocysts were washed in vitrification solution for less than 1 min at 39 °C. Four or five blastocysts/drop were then vitrified in a 1–2 μL drop of vitrification solution by releasing the drop onto the surface of a steel cube covered with aluminum foil and partially immersed in LN_2, maintaining its temperature at approximately −180 °C. The drop containing the blastocysts was instantly vitrified, subsequently removed with cold forceps, transferred into pre-cooled 1.8 mL cryovials (Nunc, Fisher), and stored in liquid N_2 at −196 °C. The other half of the blastocysts from each of the four IVF groups (BO or

IVF-M199, for 6 or 18 h) served as controls, and were transferred into fresh culture medium (SOF + 7.5% FBS) and allowed to develop until the hatched stage at Day 9. Hatched blastocysts were stained to enable counting of the embryonic cell nuclei, as described previously. Briefly, blastocysts were stained with Hoechst 33342 (10 μg/mL with 20% glycerol in DPBS). Hatched blastocysts were mounted under a cover slip and their total cell numbers were determined by counting the number of nuclei under epifluorescent microscopy. Ten hatched blastocysts from each of the four treatment groups were utilized for cell counts.

2.3.2. Thawing of vitrified IVF blastocysts

Thawing of vitrified expanded blastocysts was performed on a warming plate (39 °C), and all thawing solutions were pre-equilibrated at 39 °C in 5% CO_2. A 1.8 mL cryovial was removed from the liquid nitrogen (LN_2) tank, and the vitrified drop containing 4 or 5 blastocysts was carefully emptied onto the surface of a steel cube that was covered with aluminum foil and partially immersed into LN_2, maintaining its temperature at approximately −180 °C. The vitrified drop with blastocysts was then plunged into 3 mL of warm (39 °C) 0.3 M sucrose for 1 min. Blastocysts recovered were immediately transferred into 0.15 M sucrose for 5 min, then rehydrated for 5 min in M199 + 20% FBS (holding medium) at 39 °C. Then, recovered blastocysts were washed twice in culture medium (SOF + 7.5% FBS) and cultured in 5% O_2, 5% CO_2 and 90% N_2 at 39 °C for 48 h, for further evaluation.

2.4. Statistical analyses

All data were arc-sine transformed and then subjected to a one-way analysis of variance (ANOVA). Differences between specific IVF media, sperm–oocytes incubation periods, survival rates, and total cell number of hatched blastocysts were determined using Bonferroni's test for pair-wise comparison of means. Significance implies a $P < 0.05$ (unless otherwise stated).

3. Results

3.1. Experiment 1

Results of in vitro fertilization, polyspermic fertilization, and blastocyst formation rates are shown in Fig. 1 and Table 2; these compare the four fertilization media used. The type of fertilization medium used did not affect the total cleavage rate. However, a highly significant proportion of polyspermic fertilization was observed in BO (7%) and in MTM (6%) compared to that in IVF-M199 (1%) or m-KSOM medium (1%). For post-fertilization embryo development, oocytes fertilized in IVF-M199 resulted in a significantly greater proportion of embryos that developed to 8-cells, as well as to the blastocyst stage (57% and 40%, respectively) than did those in MTM (26% and 22%, respectively) or m-KSOM (14% and 18%, respectively). Furthermore, oocytes fertilized in either IVF-M199 or in BO produced a comparable proportion of embryos developing to blastocyst and hatching at Day 9 (40% and

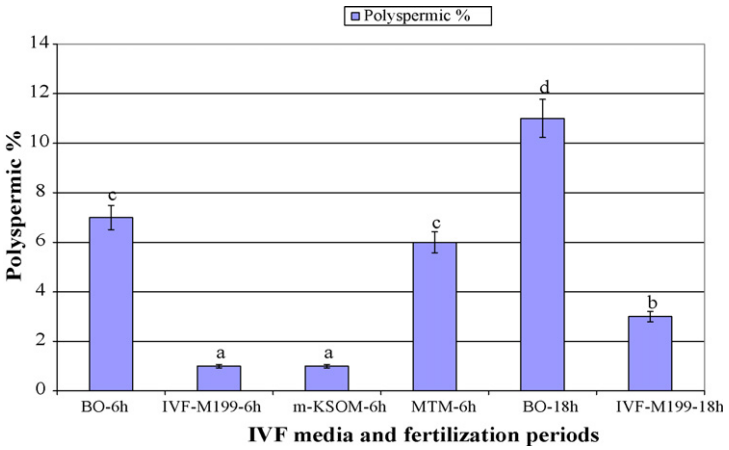

Fig. 1. Effect of IVF duration and media on polyspermic fertilization of bovine oocytes matured and fertilized in vitro. After 6 or 18 h of fertilization (BO-6 h or BO-18 h; IVF-M199-6 h or IVF-M199-18 h), 10 pooled presumptive zygotes were collected from each fertilization medium in Experiment 1 (replicated 8 times per treatment groups, $n = 320$) or Experiment 2 (BO-6 h, $n = 80$; BO-18 h, $n = 80$, IVF-M199-6 h, $n = 80$; IVF-M199-18 h, $n = 80$) and fixed to evaluate polyspermic fertilization. The percentage of polyspermic fertilization was calculated based on the total number of oocytes per treatment group. [a–d]Values with different superscripts across IVF treatment groups are different ($P < 0.05$).

Table 2
Mean (±S.E.M.) fertilization rates and embryo development to hatching for four sperm wash and IVF media

IVF media	Oocytes (*n*)	Cleavage rates 48 h post-fertilization		Day 8 Total blastocysts (%)	Day 9 Hatched (%)
		≥2 cells (%)	8-cells (%)		
BO	415	82 ± 3 a	31 ± 4 a	35 ± 5 a	74 ± 6 ab
IVF-M199	413	77 ± 2 a	57 ± 3 b	40 ± 4 a	79 ± 6 a
m-KSOM	410	58 ± 3 b	14 ± 4 c	18 ± 3 b	58 ± 4 c
MTM	417	75 ± 4 a	26 ± 5 a	22 ± 5 b	66 ± 7 bc

Within a column, values with different letters a–c differ ($P < 0.05$). BO = Brackett and Oliphant's medium, IVF-M199 = modified medium 199 for IVF, m-KSOM = modified potassium simplex optimization medium, and MTM = modified Tyrode's medium. The proportion of cleavage, and blastocyst rates were calculated by dividing the number of cleaved embryos, and blastocysts by the total number (*n*) of oocytes used. Hatched blastocysts by total number of blastocysts in respective treatment groups.

79%, respectively versus 35% and 74%, respectively, Table 2). Fertilization in BO medium, widely used for bovine IVF, was superior to that in either m-KSOM or MTM for subsequent blastocyst formation. More importantly, the oocytes fertilized in m-KSOM resulted in the lowest rate of development to the blastocyst stage (18%).

3.2. Experiment 2

Results on polyspermic fertilization and blastocyst formation rates are shown in Fig. 1 and Table 3. Total cell numbers of hatched blastocysts post-vitrification are given in Fig. 2. Although the results showed no clear differences in the overall cleavage rates among the treatment groups (BO 6 or 18 h versus IVF-M199 6 or 18 h), the highest rate of polyspermic fertilization (11%) was observed in oocytes incubated with sperm in BO medium for 18 h (Fig. 1). In vitro fertilization in BO for 18 h also resulted in a greater proportion of zygotes developing into 8-cell embryos (60%) when compared to BO for 6 h (38%). Surprisingly, oocytes fertilized in BO for the prolonged IVF period (18 h) resulted in the lowest rate of blastocyst formation at Day 7 (16%) and Day 9 (20%), as compared to other IVF groups (Table 3). There was no statistical difference in the proportion of blastocysts formed by Day 7 in the BO 6 h group as compared to both of the IVF-M199 groups. However, overall blastocyst formation was significantly higher (49%) from oocytes fertilized for 18 h in IVF-M199 compared to the other three IVF groups (Table 3).

The second part of Experiment 2 was to test the post-vitrification viability of blastocysts derived from 6 or 18 h incubation in BO or IVF-M199. This was accomplished by first vitrifying, then thawing the blastocysts (results are shown in Table 4). Neither the fertilization medium nor the duration of IVF altered the re-expansion and development of vitrified/thawed blastocysts after 6 and 24 h of post-thawing culture. However, by 48 h post-thawing, significant differences were evident (Table 4). In that regard a greater proportion of blastocysts developed, and subsequently hatched when they were derived from oocytes fertilized in IVF-M199 for 6 h (73% and 71%, respectively), or IVF-M199 for 18 h (79% and 76%, respectively), and similarly, from BO for 6 h (70% and 70%, respectively) than did those derived from 18 h in BO (59% and 58%, respectively). Furthermore, the extended fertilization period (18 h) in IVF-M199 did not reduce the survival rates of vitrified/thawed blastocysts (Table 4). As expected, vitrification significantly reduced survival rates of blastocyst in both the BO and IVF-M199 groups, as compared to non-vitrified control groups (Table 4). Total cell number of vitrified/

Table 3
Mean (±S.E.M.) fertilization rates and blastocyst development following incubation of bovine sperm and oocytes for 6 or 18 h in BO or IVF-M199

IVF media	Incubation period (h)	Oocytes (*n*)	Cleavage rates 48 h post-IVF (%)		Blastocyst (BL) development (%)	
			≥2 cells	8-cells	Day 7 BL	Day 9 total BL
BO	6	260	85 ± 2 a	38 ± 3 a	30 ± 4 b	35 ± 3 a
	18	258	83 ± 3 a	60 ± 4 b	16 ± 3 a	20 ± 4 b
IVF-M199	6	253	74 ± 1 a	57 ± 2 b	34 ± 2 b	38 ± 3 a
	18	266	80 ± 2 a	61 ± 3 b	36 ± 2 b	49 ± 4 c

Within a column, values with different letters a–c differ ($P < 0.05$). The proportion of cleavage, and blastocyst rate were calculated by dividing the number of cleaved embryos, and blastocysts by the total number (*n*) of oocytes used. Hatched blastocysts by total number of blastocyst in respective treatment groups.

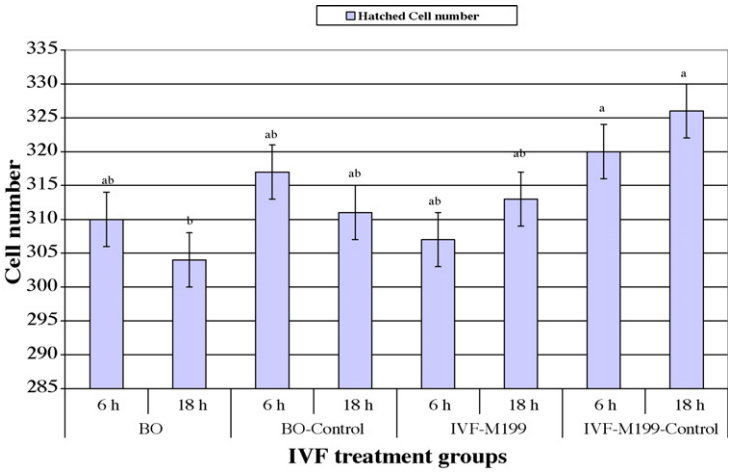

Fig. 2. Average cell number of vitrified/thawed blastocysts that re-expanded and developed to hatching following fertilization of matured bovine oocytes in BO medium (BO-6 h or BO-18 h) or IVF-M199 (IVF-M199-6 h or IVF-M199-18 h) for a 6 or 18 h period. Hatched blastocysts were mounted under a cover slip and stained with Hoechst 33342. Their total cell numbers were determined by counting the number of nuclei under epifluorescent microscopy. Ten hatched blastocysts per each of the four treatment groups in Experiment 2 were utilized for cell counts. The average number of cells was calculated based on the total number of blastocysts per treatment groups. [a,b]Values with different superscripts across IVF treatment groups are different ($P < 0.05$).

thawed blastocysts (Fig. 2) that re-expanded and developed to hatching from IVF-M199 for 6 or 18 h (307 ± 4 and 313 ± 3, respectively) and from BO for 6 h (310 ± 5) were similar to those re-expanded/hatched blastocysts derived from 6 or 18 h IVF in the non-vitrified control groups. Blastocysts that developed from either 6 or 18 h IVF in IVF-M199 or BO control groups (non-vitrified) had between 311 ± 4 and 326 ± 3 cells (Fig. 2). However, a significantly lower number of total cells (304 ± 5) were observed in the vitrified/warmed blastocysts that hatched following 18 h of IVF in BO.

4. Discussion

In this study, a high fertilization rate was achieved using frozen-thawed sperm washed and incubated with matured oocytes in modified M199 (IVF-M199), thus, confirming the ability to successfully fertilize matured oocytes in vitro with a low incidence of polyspermy. Additionally, a greater proportion of the zygotes fertilized in IVF-M199 developed into 8-cell embryos 6 h post-fertilization than in the other three IVF media tested. In the present study, development up to the blastocyst stage was somewhat dependent upon the type of medium used for preparing frozen-thawed sperm, and used for its incubation with oocytes in vitro. Modified M199, routinely used for oocyte maturation and embryo culture, was comparable to BO medium, commonly used for IVF, in terms of promoting blastocyst formation. Moreover and of greater interest to researchers and the IVF industry, was that prolonging the duration of IVF to 18 h in IVF-M199 resulted in better quality blastocysts that demonstrated cryotolerance with a higher hatching rate than those from an 18 h BO incubation (Table 4). Possibly of even greater

Table 4
Re-expansion and development of vitrified/thawed blastocysts derived from matured oocytes fertilized in BO or IVF-M199

IVF media groups	Incubation period (h)	Blastocysts (*n*)	Post-warming blastocyst development			
			6 h (%)	24 h (%)	48 h (%)	Hatched (%)
BO control (non-vitrified)	6 or 18	89	100 ± 5 a	97 ± 6 a	95 ± 6 a	82 ± 5 a
IVF-M199 control (non-vitrified)	6 or 18	96	100 ± 4 a	100 ± 5 a	98 ± 4 a	83 ± 4 a
BO (vitrified)	6	116	77 ± 3 b	77 ± 3 b	70 ± 5 c	70 ± 6 b
	18	77	74 ± 2 b	68 ± 2 b	59 ± 4 b	58 ± 5 c
IVF-M199 (vitrified)	6	114	82 ± 2 b	78 ± 3 b	73 ± 4 c	71 ± 5 b
	18	122	85 ± 2 b	85 ± 2 b	79 ± 2 c	76 ± 3 ab

Within a column, values with different letters a–c differ ($P < 0.05$). The proportion of post-thawing development was calculated by dividing the number of blastocysts, which survived by the total number (*n*) of blastocysts vitrified.

relevance was that these blastocysts had total cell numbers that were comparable to those of fresh controls (non-vitrified; Fig. 2). In this study, a prolonged sperm–oocyte incubation period (18 h) in the traditional fertilization medium, BO medium, resulted in the lowest blastocyst formation rate (Table 3), which was consistent with the reported observations by Fukuda et al. [2], albeit our cleavage rate was not altered by fertilization medium or incubation period. Consequently, this study confirmed that using BO medium for fertilization requires the removal of oocytes after 6 h of IVF, as reported previously [1–3], likely due to the presence of dead and dying sperm.

It is well known that M199 is a more complex medium, routinely used for oocyte maturation (IVM-M199), whereas BO medium is a relatively simple formulation. Therefore, utilizing a modified M199 for IVF (IVF-M199), oocytes are exposed to fewer osmotic changes, and variations in their environment are minimized during the change from IVM-M199 to IVF-M199. Otherwise, the alteration to the oocytes' environment is considerably more drastic when changing from IVM-M199 to IVF in BO, m-KSOM or MTM. Moreover, incubating oocytes and sperm in IVF-M199 for up to 18 h resulted in less incidence of polyspermic fertilization (Fig. 1), and did not adversely affect the fertilization rate or the in vitro development of bovine embryos. The incidence of polyspermic fertilization has been reported to increase with extended sperm–oocyte incubation periods, and the rate of blastocyst formation has been shown to decrease as well, although, the magnitude of these effects may vary from one bull to another [23,24]. Our study concurs with these observations, and when BO medium was used for a prolonged (18 h) IVF period, a higher incidence of polyspermic fertilization, and the lowest rate of blastocyst development were recorded as compared to an extended (18 h) IVF in M199. Our observations were consistent with the findings by Long et al. [25] and Rehman et al. [26].

Although KSOM is frequently used for embryo culture [17], it was the worst fertilization medium (m-KSOM) tested in this study, and had the lowest ($P < 0.05$) cleavage rates and the poorest embryo development. In contrast, IVF in MTM resulted in a high incidence of polyspermic fertilization (Fig. 1), but with a similarly poor rate of embryo development to the blastocyst stage. The factor(s) that contributed to the poor fertilization rates achieved in the m-KSOM IVF group is not known and beyond the realm of this study. However, lower concentrations of NaCl and $NaHCO_3$ in m-KSOM and MTM, as compared to BO or IVF-M199, might have played a role in the differences observed (Tables 1 and 2). Kim et al. [27] suggested that the ability of bovine sperm to fertilize appears to be more stable in higher, rather than lower concentrations of NaCl, and high NaCl concentrations have also been reported to be beneficial for sperm penetration in the rat [28].

Further differences observed in the efficacy of IVF-M199 (Table 1) compared to the other IVF media (BO medium, m-KSOM, and MTM) tested in this study may have been caused by the presence of antioxidants, such as glutathione and L-ascorbic acid, that are contained in IVF-M199 (Table 1) and could play a role in protecting oocytes and sperm during IVF. Antioxidants are essential for protecting frozen-thawed sperm, as well as oocytes, against damage from oxidative stressors, including hydrogen peroxides [29]. The ability of bovine semen to defend itself against oxidative stress has been shown to be decreased by the freeze-thawing process [29,30]. In addition, reactive oxygen species, such as hydrogen peroxide, lower the motility of bovine sperm [31], and decrease a sperm's ability to penetrate an oocyte; this type of stress has also been linked to the blocking of sperm–oocyte fusion in mice [31,32]. In IVF-M199, oxidative stressors have been neutralized by the presence of glutathione and L-ascorbic acid; for this reason, there should be less damage to oocytes/zygotes and enhanced embryo development when IVF-M199 is used for both sperm wash and fertilization medium. Furthermore, it would appear that the use of in vitro fertilization media containing antioxidants is beneficial to preserving a sperm's fertility. It should be noted that semen from different individual bulls may have different rates of fertilization depending on the type of IVF medium used, and there is varying ability among sperm from different bulls to become capacitated [33]. Therefore, several areas of research remain of great interest, including the effects of antioxidants in IVF media, the role played by IVF media with sperm from various bulls, and the implications of media in improving fertilization efficiencies with sex-sorted semen.

In Experiment 2, vitrification was used as a method of assessing the viability of blastocysts derived from oocytes fertilized in either BO or IVF-M199 for periods of 6 or 18 h. Prior to this study, the contribution of IVF medium and the duration of fertilization to the quality of blastocysts, and their ability to endure cryopreservation had not been examined. Cryopreservation of bovine embryos is a necessary step in many applications and has been frequently used to validate the viability of blastocysts produced in vitro [34]. Matured bovine

oocytes, fertilized in IVF-M199 for a prolonged IVF period of 18 h, developed better (8-cell embryo and blastocyst formation), and had enhanced viability that was demonstrated by post-cryopreservation (vitrification) survival and total cell number, as compared to those fertilized in BO medium for the same extended interval. It is reasonable to assume that the rate at which embryos developed to blastocysts, following fertilization in IVF-M199, was enhanced because they were of better quality. The kinetics of zygotic development to the 8-cell stage was higher in oocytes fertilized in IVF-M199, and early cleavage rates have been reported to be one of the possible factors influencing blastocyst formation, quality, and post-thaw survival [34–37]. McKiernan and Bavister [38] showed that hamster embryos that developed faster in vitro were twice as likely to develop into viable fetuses. Additionally, embryos that develop at a faster rate appeared to be more tolerant to cooling [37], although, this observation was in contrast with our results observed for the BO 18 h group. As expected, vitrification reduced the survival rate of blastocysts, compared to non-vitrified control groups, regardless of the IVF conditions (Table 4).

In conclusion, IVF-M199 was successfully utilized for IVF, compared favorably to BO medium, offered the advantage of an extended IVF period for up to 18 h, and resulted in better quality blastocysts with increased cryotolerance.

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